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## Antitumor activity with no toxicity of propolis from Meghalaya, India in ascites Dalton's lymphoma-bearing mice

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Propolis or bee glue is an important natural bee product that has been used by humans to meet the needs of health since ancient times. Despite its many valuable uses, scientific research on propolis is still limited. Despite rich traditional zootherapeutic knowledge of the people and affluent biodiversity of North-east India, almost no research study has been undertaken on the assessment of various biological properties of propolis from this region. Recently, the antibacterial and antitumor activity of the methanolic extract of propolis (MeOH-propolis) from Meghalaya was reported by us only. The present study was undertaken to further examine the antitumor activity and toxicity in ascites Dalton's Lymphoma bearing mice itself and compare with that of cisplatin, a well established anticancer drug also known to cause different toxicities in the host. MeOH-propolis showed potent anticancer activity against ascites Dalton's lymphoma. The assessment of various toxicity parameters such as hematotoxicity, nephrotoxicity, hepatotoxicity and sperm abnormality revealed that MeOH-propolis treatment did not develop any toxicity while cisplatin developed these toxicities in the host. It is suggested that the increasing antioxidative defense activity of the MeOH-propolis could be involved in its ability to avoid any toxicity in the host. The findings from the present study showing the combined action of MeOH-propolis as anticancer as well as its ability to stabilize various toxicity parameters in cancer-bearing mice suggest the possibility of using propolis as a natural alternative to chemotherapy to shun the side effects.

**Keywords:** Cisplatin, Dalton's lymphoma, Propolis, Sperm abnormality, Toxicity.

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### Introduction

Cancer is the second leading cause of death globally and is responsible for an estimated 9.6 million deaths in 2018<sup>1</sup>. Chemotherapy has proved to be an effective treatment that can be used either singly or in combination with surgery and/or radiotherapy against various types of cancers. However, the efficacy of most of the anticancer drugs is often hampered by the development of various side effects<sup>2</sup> and acquired resistance by cancer cells<sup>3</sup>. Therefore, in an attempt to overcome the side effects of chemotherapeutic drugs while maintaining therapeutic efficacy, the primary focus of the researchers has also been directed towards natural resources involving plants, animals microorganisms<sup>4,5</sup>.

Animals or animal products have been used in the treatment of various diseases including cancer<sup>6,7</sup>. Honeybees, a social insect, belong to the order-Hymenoptera and family-Apidae<sup>8</sup>. From honeybees,

seven amazing products, i.e., bee pollen, bee wax, royal jelly, honey, mead, bee bread, and propolis have been identified which have proved to be useful to humankind and treating with honeybee products is called apitherapy<sup>9</sup>. Out of these products, propolis is of vital importance for the survival of the honey bees in the beehive and also to people. Etymologically, the Greek word propolis means a *pro*, for or in defence, and *polis*, the city, that is "defence of the city (beehive)"<sup>10</sup>. Propolis, also known as 'bee glue' is the lipophilic, brownish-coloured sticky natural substance of beehive which is used as a sealant and sterilant in and around the beehive<sup>11</sup>. Honeybees collect resinous sticky material with their hind legs from leaf buds, twigs and trunk wounds of various plants, mix it with salivary secretions and wax in the beehive colony to form propolis<sup>12,13</sup>. Today propolis is widely used in medicine, cosmetics, and food industries due to its versatile biological and pharmacological activities such as antibacterial, anticarcinogenic, antiviral, immunomodulatory, anticancer, anti-inflammatory and antidiabetic properties<sup>11,14,15</sup>. The antiproliferative

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effects of propolis, caffeic acid phenethyl ester (CAPE) and chrysin in cancer cells are the result of the suppression of complexes of cyclins, as well as cell cycle arrest, induction of apoptosis pathways<sup>16</sup> and activation of p53 tumor suppressor protein<sup>17</sup>.

The hepatoprotective<sup>18</sup>, pancreatoprotective<sup>19</sup>, radioprotective<sup>20</sup> and reno-protective<sup>21</sup> properties of propolis have been reported. The composition and constituents of propolis vary depending on the geographical vegetation, climate, honey bee species as well as by the collecting season<sup>11,22</sup>. Very often the name of propolis is based on the place and country of origin such as Turkish propolis<sup>13</sup>, Indian propolis<sup>15</sup>, Nigerian propolis<sup>19</sup>, Brazilian propolis<sup>23</sup>, Iranian propolis<sup>24</sup>, Algerian propolis<sup>25</sup>.

Meghalaya is one of the eight states of North-east India, known for its clouds and the highest rainfall in the world. It comprises 11 districts and the population is predominantly tribal which mainly includes Khasis, Garos, and Jaintias who have their respective dialect, distinct ways of life, belief, traditions, and cultural heritage<sup>26</sup>. In the present study, the propolis was collected from Ngunraw village of Meghalaya, so it is being referred here as Meghalaya propolis. Ngunraw village is approximately 135 Km away from Shillong, situated between 25°17'52.5" N latitude and 91°19'23.2" E longitude located under Mawkyrwat block of South West Khasi Hills district, Meghalaya. Agriculture and apiculture are the main sources of livelihood of the people and the Indian hive-bee, *Apis cerana indica* (Apidae) is the common honey bee species reared in this area.

There is almost no study undertaken on the assessment of the biological properties of propolis from North-east India. Given a variety of reports on the properties of propolis from different parts of the world, the antibacterial and antitumor potential of propolis from Meghalaya was studied for the first time by us<sup>27</sup>. Very often the assessment of the toxicity of an anticancer drug is studied by the treatment of normal animals which does not give the real circumstances because it is the tumor-bearing animals, not the normal animals, which are treated with these drugs. Therefore, the present study was undertaken to look at the effect of this propolis extract on the antitumor activity and toxicity concerning histopathology of liver and kidney, and haematological parameters in the same Dalton's lymphoma tumor-bearing mice. Cisplatin

(*cis*-diamminedichloroplatinum-II), a well established anticancer drug also known to cause different toxicities, was used as the reference drug for a comparative elucidation of the propolis-mediated findings.

## Materials and Methods

### Chemical

Reduced glutathione (GSH), 5, 5 N-dithiobis-2-nitrobenzoic acids (DTNB), 1-chloro-2,4-dinitrobenzene (CDNB), and bovine serum albumin (BSA) were purchased from Sigma Chemical Co., St. Louis, Mo, USA. Ethylenediamine tetra-acetic acid (EDTA), and other chemicals used in the experiments were of analytical grade and purchased from SRL Pvt. Ltd., Mumbai, India. Cisplatin solution (1 mg per mL of 0.9%, NaCl) was obtained from Biochem Pharmaceutical Industries, Mumbai.

### Animal and tumor maintenance

Swiss albino mice colony is being maintained under conventional laboratory conditions at room temperature of 22±2 °C keeping 5-6 animals per propylene cage using paddy husk as a bed with food pellets (Amrut Laboratory animal feeds, New Delhi) and drinking water *ad libitum*. Inbred Swiss albino mice of both sexes in the age group of about 10-12 weeks weighing about 28-30 g were used for the experiments. Dalton's lymphoma is a transplantable and highly invasive T cell lymphoma that develops an ascitic tumor in murines<sup>28</sup>. Ascites Dalton's lymphoma has been widely used by the researchers for evaluating the anticancer potentials of a variety of drugs. Dalton's lymphoma (DL) is being maintained *in vivo* in mice of both sexes by serial intraperitoneal (i.p.) transplantation of approximately 1x10<sup>7</sup> viable tumor cells per animal (0.25 mL in phosphate-buffered saline (PBS), pH 7.4). Tumor transplanted hosts usually survive for 19-21 days. The maintenance, use of the animals and the experimental protocol of the present study have been approved with reference number IEC/NEHU/December 04/2014 by the Institutional ethics committee (animal models), North-Eastern Hill University, Shillong, India

### Propolis collection and preparation of methanolic extract

Beehives maintained in an apiary at Ngunraw village, South West Khasi Hills district of Meghalaya, India were collected during spring and winter seasons. It was cleaned, freed from wax and raw propolis was separated. Its methanolic extract (MeOH-propolis) was prepared as per the details described earlier<sup>27</sup>.

### Drug treatment and antitumor activity

As per the earlier report, the dose and schedule of MeOH-propolis<sup>27</sup> and cisplatin<sup>29</sup> treatment of tumor-bearing mice was selected. Tumor-transplanted mice were randomly divided into three groups consisting of 10 mice in each group and the day of tumor transplantation was taken as day zero. Group-I mice served as tumor-bearing control and received extract vehicle only. Group-II mice were administered with methanol extract of propolis (MeOH-propolis) (i.p., 50 mg/kg body weight) for 5 consecutive days starting from the 6<sup>th</sup> day post-tumor transplantation. Group-III mice were administered with the reference drug, cisplatin (i.p., 2 mg/kg body weight/ day.) for five consecutive days starting from the 6<sup>th</sup> day post-tumor transplantation.

The death of animals, if any, and body weights of animals in different groups were recorded daily. The antitumor efficacy was reported in percentage of the average increase in life span (% ILS) calculated using the formula:

$$\text{Percentage Increase in life span} = \frac{\text{MST of treated group} - \text{MST of control group}}{\text{MST of control group}} \times 100$$

$$\text{Where MST (Mean survival time)} = \frac{\sum \text{survival time (days) of each mice in a group}}{\text{Total number of mice}}$$

After 48 and 96 h of treatment i.e., on 12<sup>th</sup> and 14<sup>th</sup> day post tumor transplantation, the animals were sacrificed by cervical dislocation, tissues and blood were collected, and used for some biochemical, histological and haematological investigation. For comparative analysis, haematological study was also done on blood from normal mice i.e., mice without tumor and any treatment.

### Viability of DL cells and splenocytes

Trypan blue exclusion assay was carried out to check the viability of DL cells and splenocytes collected from the mice under different treatment conditions<sup>30</sup>. It is based on the principle that viable cells possess intact cell membranes that exclude trypan blue, whereas dead cells do not. Therefore, all the viable cells will have a clear cytoplasm whereas a nonviable cell will have a blue cytoplasm. Dalton's lymphoma ascites and spleen were collected from the mice in different groups at 96 h of treatment. The ascites tumor was centrifuged gently (800 × g, 10 min at 4 °C). The cell pellets were resuspended in PBS to get uniform cell suspension. The spleen was minced into small pieces with a scissor, squeezed

between two microscopical slides and the detached cells were drained gently in PBS to get the single-cell suspension. To lyse the erythrocytes, an equal volume of 0.85% NH<sub>4</sub>Cl was added to the cell suspension, mixed gently and incubated at room temperature for 3-4 minutes. After centrifugation (400×g at 4 °C for 10 min), the pelleted cells were resuspended in PBS to get the single-cell suspension of splenocytes which were used to determine viability. To the cell suspension, an equal volume of 0.4% trypan blue dye (4 mg/100 mL PBS) was added and incubated at room temperature for 4-5 min with intermittent gentle shaking. After 5 minutes, cell suspension was taken on a hemocytometer and counted under a light microscope. The percentage of dead cells i.e., cytotoxicity was calculated by dividing the number of dead cells by the number of total cells and multiplying by one hundred.

### Reduced glutathione (GSH) estimation

Total reduced glutathione (GSH) in liver, kidney and DL cells was determined using the method of Sedlak and Lindsay<sup>31</sup>. Briefly, 5% tissue homogenates of DL cells, kidney, or liver were prepared in 0.02 M EDTA (pH 4.7). Total GSH was determined by adding the tissue homogenate or pure reduced form of glutathione (100 µL) to 0.9 mL of 0.02 mol/L EDTA, pH 4.7 and 1 mL of 0.2 mol/L Tris-EDTA buffer, pH 8.2, and followed by 20 µL of Ellman's reagent (10 mmol/L DTNB in methanol). After 30 minutes of incubation at room temperature, the reaction mixture was centrifuged at 3000 × g and the absorbance of the clear supernatant was read against a reagent blank at 412 nm in a Varian Carey-50 spectrophotometer. The results were read from a standard curve prepared from 1mmol/L solution of reduced glutathione.

### Evaluation of hematological parameters

The blood for haematological studies was collected from the eye orbit using the capillary tube from the mice in different experimental groups into a sterilized tube containing anticoagulant heparin (15-20 IU per mL of blood) and used for the haematological study. The haemoglobin content, red blood cells (RBC) counts, white blood cells (WBC) counts, and packed cell volume (PCV) were determined according to the method described by Dacie and Lewis<sup>32</sup>.

### Haemoglobin (Hb) estimation

Freshly collected blood was diluted (200 times) with cyanide-ferricyanide reagent (200 mg potassium ferricyanide and 50 mg potassium cyanide in 1 litre

of distilled water, pH 9.6) and allowed to stand for 10 min at room temperature. The absorbance was read at 540 nm in a spectrophotometer. The haemoglobin content was calculated as follows:

$$\text{Hb concentration (g/dL)} = A_{540} \times 64500 \times \text{dilution factor} / 44.0 \times d \times 1000 \times 10$$

where,  $A_{540}$  = absorbance at 540 nm, 64500 = molecular weight of Hb, dilution factor = 200, 44.0 = millimolar extinction coefficient,  $d$  = layer thickness (cm), 1000 = conversion factor for mg to g, 10 = conversion factor for g/L to g/dL.

#### **Red blood cells (RBCs) counts**

Freshly collected blood was diluted (200 times) with RBC diluting fluid. The diluted blood sample was filled in a Neubauer counting chamber with its cover-glass already in position and the RBC counting was done and calculated as follows:

$$\begin{aligned} \text{RBC count per } \mu\text{L} &= N \times 1 \times 200 (\text{dilution}) / 0.02 \\ &= N \times 10^4 \times 10^6 / \text{L} \end{aligned}$$

#### **White blood cells (WBCs) counts**

Freshly collected blood was diluted (20 times) with white blood cells (WBC) diluting fluid (2% acetic acid) contained in a plastic tube. The diluted blood sample was mixed for about 1 minute and counted in a Neubauer chamber. The number of white blood cell was calculated as follows:

$$\begin{aligned} \text{WBC count per } \mu\text{L} &= N \times 10 \times 20 (\text{dilution}) = N \times 200 \\ &= N \times 200 \times 10^6 / \text{L} \end{aligned}$$

#### **Packed cell volume (PCV) determination**

Blood was collected in a wintrobe tube and centrifuged for 30 min (2000 x g, at 4 °C). The height of the column of red cells was taken as the PCV (the volume occupied by the red blood cells expressed as a fraction of the total volume of the blood).

#### **Differential leucocytes counts**

Differential leucocytes count was carried out according to Swarup *et al.*<sup>33</sup>. A drop of fresh blood was taken on a clean slide and a thin and uniform blood film was prepared with the help of another clean slide. The blood film was air-dried for overnight, stained with Leishman's stain the following day and mounted in DPX. Counting was done under the microscope in a narrow longitudinal strip of the blood film starting from one end of the film to the other end. The number of different types of white blood cells (neutrophils, basophils, monocytes, lymphocytes, and eosinophils) was noted and expressed in percentage.

#### **Scanning electron microscopy (SEM) study for the abnormality in RBCs**

A small drop of freshly collected blood was taken in a clean micro centrifuged tube and fixed in 2.5% glutaraldehyde for 30 min at room temperature. After fixation, the blood was centrifuged for 5 min at 750 x g at 4 °C. The pellet was washed twice in phosphate buffer and re-suspended in distilled water. A thin film of suspension was made on a clean coverslip and allowed to air dry. The coverslip was mounted on a brass stub with double-stick tape, coated with gold in a fine coat ionic sputter (SCD-005, BAL-TEC Co.). The red blood cells (RBCs) were thoroughly examined for abnormalities in RBCs under an electron microscope (JEOL JSM-6360, SEM) and photographed.

#### **Histopathological study**

The liver and kidney were collected from the mice in different experimental groups after 7 days of the last treatment and fixed in Bouin's fixative until used. The tissues were washed with tap water and processed for dehydration in progressively increasing concentrations of ethanol (30, 50, 70, 90, and 100%), cleared in xylene, infiltrated by liquid paraffin and cooled to form a solid paraffin block containing the tissues. Paraffin-tissue sections (5-6  $\mu\text{m}$  in thickness) were cut with a microtome (Sipcon) and mounted on clean glass slides which had been smeared with a drop of Mayer's albumin. It was then spread on a hot plate at about 50 °C for 2-3 minutes. The sections were dehydrated and stained with hematoxylin-eosin, cleared in xylene and mounted with cover glass, examined and photographed under an optical microscope DMRX (Leica 650) to assess different structural features in the tissue.

#### **Sperm abnormality assay**

The male mice in different groups were sacrificed on the 10<sup>th</sup> day of treatment. The cauda epididymis was removed and placed in physiological saline. It was then minced into pieces and kept undisturbed for 20 minutes. The sperms were spread on a clean slide, fixed in absolute methanol for 15 minutes and then stained with 1% aqueous eosin-Y on the following day. Five hundred sperms from the mouse in each group were examined for the abnormalities in sperm head and tail shapes following the criteria as close as possible to those established by Wyrobeck and Bruce<sup>34</sup>.

#### **Statistical analysis**

The results were expressed as mean  $\pm$  S.D. The data were statistically analyzed using one-way analysis of

variance (ANOVA) with Tukey's multiple comparison post hoc tests to compare the level of significance between control and experimental groups. A  $P$ -value  $<0.05$  was considered statistically significant in all cases.

## Results

### Viability of DL cells and splenocytes

Trypan blue exclusion test for the cell viability showed that MeOH-propolis, as well as cisplatin treatment, caused a significant decrease in the viability of DL cells. However, splenocytes' viability was not affected by MeOH-propolis treatment (Fig. 1).

### Antitumor activity

Following DL tumor transplantation, the increase in belly size with the sluggish movement of the animals was noted from the 3-4<sup>th</sup> day onwards showing an early sign of tumor development. Control tumor-bearing mice showed a gradual increase in

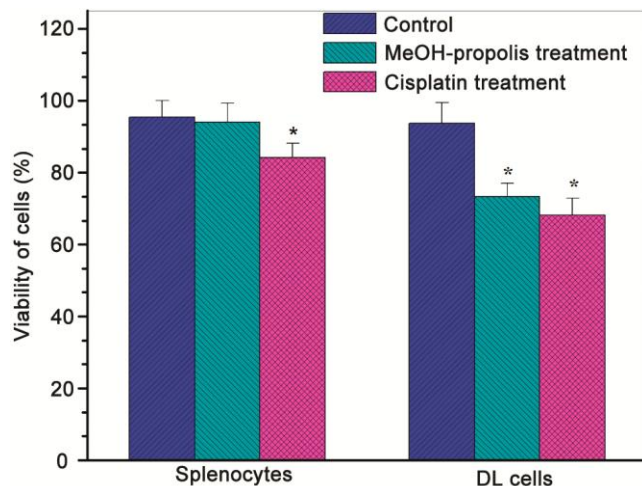


Fig. 1 — Trypan blue exclusion viability evaluation of Dalton's lymphoma (DL) cells and splenocytes from tumor-bearing mice. Control= DL cells treated with drug vehicle alone. A significant decrease in the viability of DL cells was observed after the methanol extract of propolis (MeOH-propolis) and cisplatin treatment. The results are expressed as mean $\pm$ S.D.,  $n=4$ . The significance of the changes between control and the treated group was tested by ANOVA,  $p \leq 0.05$ .

body weight until death while MeOH-propolis or cisplatin treatment significantly prevented the increase in body weight due to tumor growth (Fig. 2). The tumor-bearing mice treated with MeOH-propolis (Group-II) or cisplatin (Group-III) showed the average increase in life span (% ILS) of about 97% and 133% respectively (Table 1).

### Reduced glutathione (GSH) content

GSH contents in the tissues of tumor-bearing control mice showed the highest level of GSH in the liver followed by kidney and DL cells. MeOH-propolis treatment of tumor-bearing mice showed a slight increase in GSH level in the liver and kidney but caused a significant decrease in GSH level in DL cells. In the case of reference drug cisplatin, the significant decrease in GSH level was observed in kidney and DL cells (Table 2).

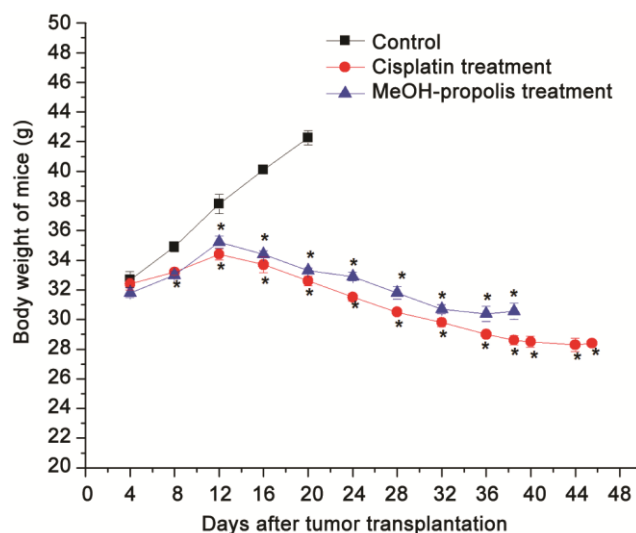


Fig. 2 — Changes in the bodyweight of tumor-bearing mice in different groups. Control= Ascites Dalton's lymphoma bearing mice treated with drug vehicle alone. A decrease in the bodyweight of the hosts was observed after the methanol extract of propolis (MeOH-propolis) and cisplatin treatment. The details of the treatment schedule are described in the methodology. The results are expressed as mean $\pm$ S.D.,  $n=4$ . The significance of the changes between control and the treated group was tested by ANOVA,  $p \leq 0.05$ .

Table 1 — Antitumor activity of MeOH-propolis and cisplatin against murine ascites Dalton's lymphoma (DL)

Treatment group	Day of treatment	Survival days (Mean $\pm$ SD)	ILS (%)
Group- I (Control)		19.5 $\pm$ 1.29	
Group- II (MeOH-propolis treatment, i.p, 50 mg/kg body weight/day)	6 <sup>th</sup> day post tumor transplantation (for 5 consecutive days )	38.5 $\pm$ 1.0*	97.43
Group- III (Cisplatin treatment, i.p, 2 mg/kg body weight/ day)	6 <sup>th</sup> day post tumor transplantation (for 5 consecutive days )	45.5 $\pm$ 1.5*	133.34

MeOH-propolis = Methanol extract of propolis; i.p. = intraperitoneal. ILS= Average increase in life span. Results are expressed as mean $\pm$ S.D. The significance of changes between control and different treated groups was tested by one-way ANOVA,  $n=4$ ,  $*P \leq 0.05$  as compared to the control. Control= Tumor-bearing mice treated with extract vehicle only.

### Haematological Studies

As compared to tumor-bearing control, cisplatin treatment caused a decrease in the haemoglobin (Hb) content, RBC and WBC count. Propolis treated mice did not decrease the lymphocytes, haemoglobin concentration, RBC and WBC counts (Table 3, 4). As compared to a corresponding control, a decrease in the PCV was noted after cisplatin treatment.

Table 2 — Changes in GSH content ( $\mu\text{mol/g}$  wet wt.) in the tissues of tumor-bearing mice under different treatment conditions

Treatment group	Liver	Kidney	DL cells
Control 48 h	11.16 $\pm$ 0.29	8.55 $\pm$ 0.57	6.26 $\pm$ 0.05
96 h	10.66 $\pm$ 0.52	8.29 $\pm$ 0.63	6.405 $\pm$ 0.12
MeOH-propolis 48 h	10.56 $\pm$ 0.10	8.22 $\pm$ 0.31	3.98 $\pm$ 0.26*
96 h	10.49 $\pm$ 0.14	8.79 $\pm$ 0.69	4.37 $\pm$ 0.09*
Cisplatin 48 h	9.12 $\pm$ 0.22*	6.44 $\pm$ 0.43*	3.40 $\pm$ 0.11*
96 h	8.52 $\pm$ 0.18*	6.12 $\pm$ 0.28*	3.48 $\pm$ 0.21*

Control= Tissues from untreated tumor-bearing hosts receiving extract vehicle alone. The methanol extract of propolis (MeOH-propolis) treatment caused a significant decrease in GSH content in DL cells while cisplatin decreased GSH in DL cells as well as in the liver and kidney. Results are expressed as mean  $\pm$  S.D. The significance of changes between treated and respective control was tested by one-way ANOVA,  $n=4$ ,  $*P \leq 0.05$  as compared to the control.

However, propolis treated mice showed a slight increase in PCV (Table 3)

### Morphological abnormalities in RBCs

SEM revealed the development of various abnormalities in RBCs in tumor-bearing mice under different treatment conditions. Normocytes (having a smooth surface), are the normal erythrocytes which are normal in size and shape. Different morphological abnormalities in RBCs observed include the microcytes (cells having a smaller diameter than the normal erythrocytes), macrocytes (having larger diameter), echinocytes (presence of uniformly serrated projections), acanthocytes (spiculated or thorn-like cells), schistocytes (small fragmented spindle-shaped cells), spherocytes (sphere-shaped cell smaller than normal RBC), stomatocytes (elongated cells with the presence of slot-like structure at the centre), ovalocytes (having oval-shaped cells) and elliptocytes (having elliptical-shaped cells) (Fig. 3). Cisplatin treatment caused a significant increase in abnormalities in RBCs. However, in MeOH-propolis treated tumor-bearing mice abnormalities in the RBCs did not increase and it was quite comparable to that of control (Fig. 4).

Table 3 — Changes in the haematological values in tumor-bearing mice under different treatment conditions

Treatment groups	Hb (g/dL)	RBC ( $\times 10^{12}/\text{l}$ )	WBC ( $\times 10^9/\text{l}$ )	PCV (%)
Normal	14.02 $\pm$ 0.88	7.72 $\pm$ 0.36	4.73 $\pm$ 0.34	45.87 $\pm$ 2.77
Control 48 h	11.40 $\pm$ 0.87	6.81 $\pm$ 0.86	8.87 $\pm$ 0.83	35.87 $\pm$ 0.76
96 h	9.97 $\pm$ 0.71	6.74 $\pm$ 0.67	10.86 $\pm$ 0.48	31.87 $\pm$ 0.31
MeOH-propolis 48 h	11.97 $\pm$ 0.73	7.22 $\pm$ 0.60	7.45 $\pm$ 0.47*	36.93 $\pm$ 0.33
96 h	13.08 $\pm$ 0.98*	7.88 $\pm$ 0.34*	9.04 $\pm$ 0.60	37.96 $\pm$ 0.44*
Cisplatin 48 h	10.06 $\pm$ 0.52	5.81 $\pm$ 0.51	8.19 $\pm$ 0.60	26.14 $\pm$ 0.66*
96 h	8.92 $\pm$ 0.68*	6.35 $\pm$ 0.95*	8.19 $\pm$ 0.29*	30.93 $\pm$ 0.46

Normal= Mice without tumor or any treatment condition; Control= Untreated tumor-bearing mice; Hb= Haemoglobin; RBC= Red blood cells, WBC= White blood cells; PCV= Packed cell volume; Results are expressed as mean  $\pm$  S.D. ANOVA,  $n=4$ ,  $*p \leq 0.05$  as compared to the corresponding control.

Table 4 — Differential leukocytes counts (mean %  $\pm$  S.D.) in the blood of normal and tumor-bearing mice under different treatment conditions

Treatment groups	Lymphocytes	Monocytes	Neutrophils	Eosinophils	Basophils
Normal	58.29 $\pm$ 5.52	3.58 $\pm$ 0.44	33.06 $\pm$ 2.72	1.72 $\pm$ 0.18	0.87 $\pm$ 0.19
Control (DL-bearing ) 48 h	26.92 $\pm$ 0.31	2.30 $\pm$ 0.17	67.85 $\pm$ 1.91	3.28 $\pm$ 0.21	1.15 $\pm$ 0.03
Control (DL-bearing ) 96 h	23.09 $\pm$ 0.53	1.86 $\pm$ 0.12	71.36 $\pm$ 1.79	4.19 $\pm$ 0.17	1.07 $\pm$ 0.01
MeOH-propolis- 48 h	31.05 $\pm$ 0.50*	2.82 $\pm$ 0.10	65.02 $\pm$ 3.95	5.26 $\pm$ 0.42*	0.64 $\pm$ 0.02
96 h	29.22 $\pm$ 0.27*	2.38 $\pm$ 0.11*	69.39 $\pm$ 3.16	5.71 $\pm$ 0.42*	0.81 $\pm$ 0.12
Cisplatin- 48 h	20.14 $\pm$ 0.82	2.64 $\pm$ 0.31	74.66 $\pm$ 1.05	4.97 $\pm$ 0.11	0.78 $\pm$ 0.20
96 h	18.19 $\pm$ 0.55*	2.78 $\pm$ 0.32*	77.89 $\pm$ 0.39	5.63 $\pm$ 0.40*	0.68 $\pm$ 0.11

Normal = Blood from the mice without tumor or treatment. Control = Blood from the tumor-bearing mice treated with extract vehicle only. DL = Dalton's lymphoma; MeOH-propolis= Methanol extract of propolis treated tumor-bearing mice; Cisplatin = Cisplatin treated tumor-bearing mice. The details of the treatment schedule have been given in Materials methods. Results are expressed as mean  $\pm$  S.D. One way ANOVA test,  $n=4$ ,  $*p \leq 0.05$  as compared to the corresponding control.



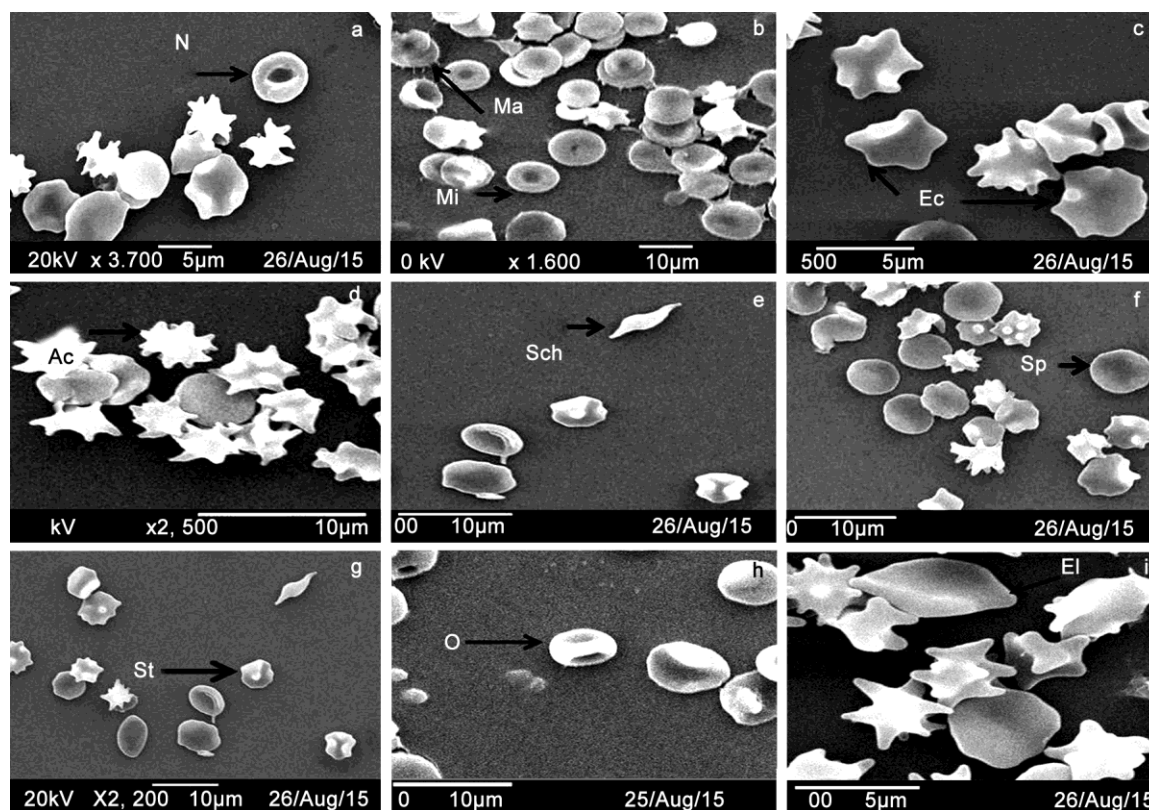


Fig. 3 — Representative scanning electron micrographs (SEM) showing normal RBCs N= Normocytes (a), and various types of abnormalities (arrows) in RBCs under different treatment conditions, Ma= Macrocytes, Mi= Microcytes (b), Ec= Echinocytes (c), Ac= Acanthocytes (d), Sch= Schistocytes (e), Sp= Spherocytes (f), St= Stomatocytes (g), O= Ovalocytes (h), El= Elliptocytes (i)

#### Histopathological studies

Histopathological examination of kidney in control mice showed normal renal glomeruli and tubules having intact epithelial cells (Fig. 5a). However, cisplatin treatment of mice caused immense destruction of the renal tubular cells represented by glomerular atrophy, infiltration of cells and tubular congestions (Fig. 5b) which supports the view that cisplatin alone treatment caused nephrotoxicity and cellular damage in the host. However, MeOH-propolis treatment of mice showed no destruction of renal tubular cells and glomerular damage thereby signifying no nephrotoxicity in the hosts (Fig. 5c).

Liver histology from control mice showed a normal arrangement of hepatocytes and proper central vein (Fig. 6a). In contrast, mice treated with cisplatin exhibited severe hepatotoxicity characterized by diffused sinusoidal distortion, congestion in the central vein and remarkable locational hepatocytes damage (Fig. 6b). However, MeOH-propolis treatment of mice showed no damage to hepatic cells indicating no hepatotoxicity (Fig. 6c).

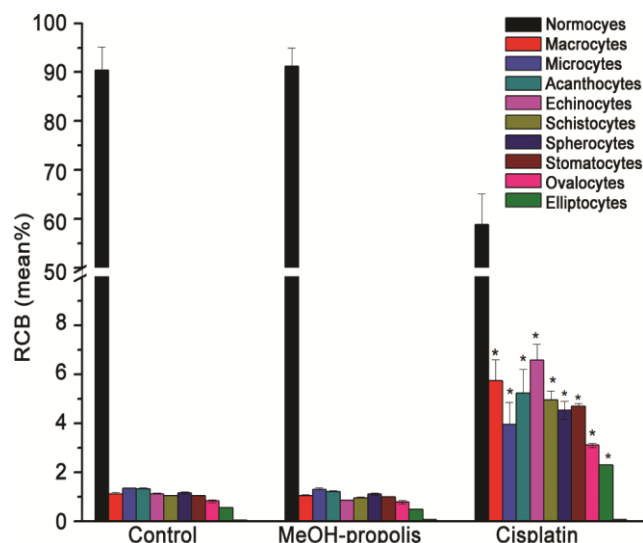


Fig. 4 — The pattern of normal and abnormal shaped red blood cells (RBCs) in the blood of control and different treatment groups. The methanol extract of propolis (MeOH-propolis) did not alter the pattern of abnormalities in RBCs while cisplatin treatment caused a significant increase in abnormalities in RBCs. Results are expressed as mean±S.D., n=3. The significance of the changes was tested by ANOVA, \* $p \leq 0.05$  as compared to control.

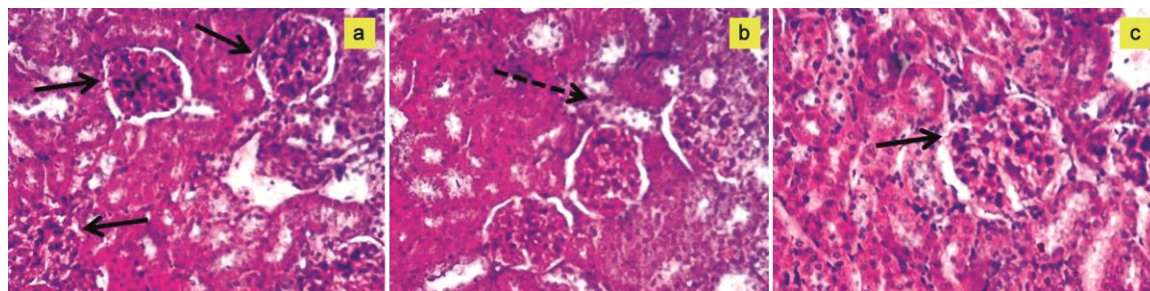


Fig. 5 — Histopathological features of the kidney from tumor-bearing mice under different treatment conditions. Histology of kidney control (a), Cisplatin (b), and MeOH-propolis treated mice (c). Regular arrows showing glomerulus and a dotted arrow showing the destruction of renal tubules, (100x magnification).

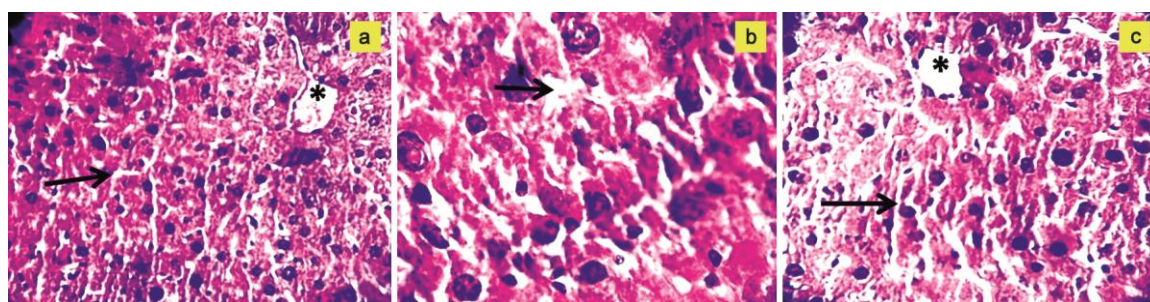


Fig. 6 — Histopathological features of liver from tumor-bearing mice under different treatment conditions. Control (a), CDDP (b), MeOH-propolis treated mice (c). Regular arrows are showing damaged hepatocytes and an asterisk marks the central vein. (100x magnification).

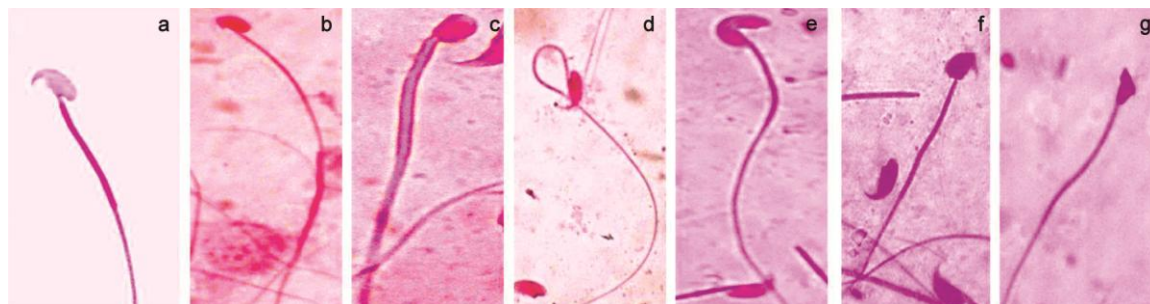


Fig. 7 — Representative photographs of various types of morphological abnormalities in sperms in Dalton's lymphoma (DL) tumor-bearing mice at different treatment conditions. (a) normal, (b) hookless, (c) balloon-like head, (d) looping mid-piece, (e) diffused head, (f) incorrect head-neck connection, and (g) amorphous head.

Table 5 — Quantitative analysis of various types of sperm abnormalities in the hosts under different treatment conditions

Treatment	No. of sperms observed	No. of abnormal sperms	HL	LMP	BH	IHNC	DH	AH	Mean % of abnormal sperm $\pm$ S.D.
Control	500	8	3	0	1	0	1	3	1.40 $\pm$ 0.2
MeOH-propolis treated	500	9	4	0	1	1	1	2	1.80 $\pm$ 0.4*
Cisplatin treated	500	37	13	3	4	2	3	12	7.4 $\pm$ 0.5*

Results are expressed as mean $\pm$ S.D. One-way ANOVA, Tukey's test,  $n=4$ , \* $p \leq 0.05$  as compared to control. HL=Hookless head, LMP=looping midpiece,

BH=balloon like head, IHNC=incorrect head-neck connection, DH=diffused head, and AH=amorphous head. Sperm analysis was carried out on the 10<sup>th</sup> day of treatment condition

#### Sperm abnormality

Cisplatin treatment induced several abnormalities in sperms. The type/shape of sperm abnormalities induced by cisplatin included hookless head, looping mid-piece, balloon-like head, incorrect

head-neck connection, diffused head, amorphous head, etc. (Table 5; Fig. 7). MeOH-propolis treatment showed very less frequency of sperm abnormalities as compared to cisplatin treatment (Fig. 8).



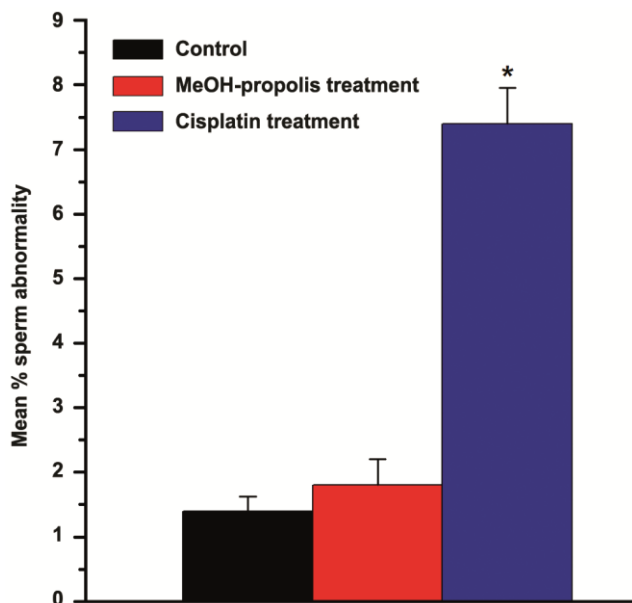


Fig. 8 — Average per cent abnormal sperms in tumor-bearing mice at different treatment conditions. Cisplatin treatment caused a significant increase in abnormalities in sperms. Results are expressed as mean $\pm$ S.D.,  $n=3$ . The significance of the changes was tested by ANOVA,  $*p \leq 0.05$  as compared to control.

## Discussion

Natural products<sup>4-6</sup> and novel natural biodegradable approach developing nanoparticles<sup>35</sup> have been used as promising sources for the discovery of new pharmaceutical agents. As a natural honeybee hive product, propolis extracts have been used as a remedial agent in traditional medicine. The composition and biological properties of propolis can vary depending on the bee species and trees and flowers they have access to<sup>12,22</sup>. The present study exhibits the findings on the assessment of anticancer activity and toxicity of methanolic extract of Meghalaya propolis (MeOH-propolis) in mice bearing Dalton's lymphoma ascites tumor which has been frequently used in the evaluation of the anti-cancer activity of various drugs and extracts derived from plants and animals<sup>6,36,37</sup>. The MeOH-propolis treatment of tumor-bearing mice did not affect the viability of the splenocytes representing normal cells while the viability of Dalton's lymphoma tumor cells was very much decreased (Fig. 1). This indicates that the MeOH-propolis has differential effects on normal cells and tumor cells. The cytoprotective role of propolis<sup>38</sup> or its chemical constituent caffeic acid phenethyl ester (CAPE)<sup>39</sup> has been reported. An increase in the survival time and changes in body weight has been used as a reliable parameter to assess

the anticancer activity of drugs<sup>36,37</sup>. As compared to the untreated DL-bearing control mice, MeOH-propolis treatment significantly prevented the increase in body weight (Fig. 2) indicating a reduction in tumor growth and resulted in increased mean survival time of the tumor-bearing mice (Table 1). Ethanol extract of Turkish propolis was reported to exhibit powerful cytotoxic effects against five human cancer cell lines<sup>13</sup>. The ethanolic extract of Moroccan propolis caused an effective reduction in the tumor volume in P815 tumor-bearing mice and apoptotic induction in P815 cells<sup>40</sup>. MeOH-propolis has also been reported to induce apoptosis in DL cells<sup>27</sup>. The reference anticancer drug cisplatin treatment also depicted a similar pattern of changes in the body weight and survival of the hosts thereby corroborating the potential anticancer activity of MeOH-propolis (Fig. 2; Table 1).

Reduced glutathione (GSH), the tripeptide of L- $\gamma$ -glutamyl-L-cysteinyl-glycine, is the most abundant antioxidant in the cells and plays a key role in maintaining intracellular redox equilibrium and in augmenting cellular defence to harmful factors<sup>41</sup>. GSH plays an important role in a multitude of cellular processes such as cell differentiation, proliferation, and apoptosis, and disturbances in GSH homeostasis are involved in the etiology and progression of many human diseases including cancer<sup>42</sup>. The modulation of the GSH-based antioxidant redox system has been suggested as a promising therapeutic strategy for overcoming cancer cell progression and drug resistance. A direct correlation between GSH levels and cellular proliferation, cancer metastatic activity as well as a decrease in the rate of cancer cell proliferation with a decrease in GSH level in cancer cells has been reported<sup>43,44</sup>. The MeOH-propolis treatment of tumor-bearing mice caused a decrease in GSH levels in DL cells but not in the liver and kidney (Table 2). The decreased GSH levels may lead to the alterations in the antioxidant machinery accompanied by decreased proliferation/survival of DL cells. The reference drug, cisplatin treatment showed a decrease in GSH level in DL cells as well as in the liver and kidney (Table 2) which may be correlated with its cytotoxicity in DL cells and toxic effects in the liver and kidney of the hosts as has been observed being described later on. Cisplatin has been well established to cause hepatotoxicity and nephrotoxicity in the hosts<sup>45</sup>. On the other hand, the observation that MeOH-propolis treatment did not decrease GSH levels in the liver and

kidney, may help to maintain normal structural and functional activity.

Hematological and biochemical parameters are important tools that aid in the elucidation of pathological/toxicological problems. Haematological findings show that as compared to normal mice, a decrease in Hb content and RBCs count occurred in control tumor-bearing mice. Chemotherapy including cisplatin treatment has been reported to cause anaemia and decrease in RBC, Hb production<sup>46</sup>. Cisplatin-treated tumor-bearing mice showed a decrease in Hb content and RBCs count (Table 3). On the other hand, MeOH-propolis treated tumor-bearing mice showed a significant increase in Hb content, RBCs count, WBCs count and hematocrit (PCV) as compared to cisplatin-treated tumor-bearing mice (Table 3). Similar to this observation an elevation of total erythrocytes counts and haemoglobin was reported in Muscovy broiler ducks kept on ethanol extracted propolis<sup>47</sup>. Other reports showed that the administration of propolis to diabetic mice significantly elevated haematological parameters such as the total number of red blood cells, haemoglobin and hematocrit level<sup>48</sup>. Similarly, the observed MeOH-propolis-mediated increase in haemoglobin content, RBCs and WBCs count may suggest its stabilizing role on haematological parameters in tumor-bearing mice. Our findings are in agreement with the report showing the combined action of Egyptian propolis as antitumor and antibacterial as well as its ability to stabilize the haematological parameters in cancer-bearing mice and it was suggested the possibility of using propolis as a natural alternative to chemotherapy to avoid the side effects<sup>49</sup>. The inclusion of Brazilian propolis in the ration of Blue-fronted Amazons has been reported to improve the haemoglobin, eosinophils and serum biochemical parameters. It was suggested that the eosinophil values are increased by the immunomodulatory action of Brazilian propolis<sup>23</sup>.

Leukocytosis is a pathological condition, usually happened due to the increased number of neutrophils and by affecting the WBCs that frequently rises after infection, chronic inflammation, and cancer. The presence of cancer itself can lead to the development of leukocytosis as a reaction against the neoplasm<sup>50</sup>. A similar observation was made here also with an increase in neutrophils, eosinophils, and basophils and decreased in lymphocytes and monocytes in control tumor-bearing mice (Table 4). Cisplatin treated

tumor-bearing mice showed a decrease in basophils and neutrophils. MeOH- propolis treatment showed a significant increase in lymphocytes and eosinophils (Table 4). The present observation is supported by another finding which showed that the ethanolic extract of propolis improved or did not change various haematological parameters in BALB/c mice<sup>51</sup>. Decreased antioxidant capacity and increased fragility in RBC are supported by the observation of various types of cisplatin-induced morphological abnormalities in erythrocytes as observed in Scanning electron microscopy of RBCs (Fig. 3, 4). On the other hand, MeOH-propolis treated tumor-bearing mice showed decreased frequency of abnormalities in the RBCs as compared to that of cisplatin (Fig. 4) which may suggest that MeOH-propolis has minimal or no hematotoxicity. Exact mechanisms underlying propolis-mediated stabilization in haematological values are not known, however, it may be suggested that the observed improvement in haematological values may be involved in enhancing host immunity, thereby increasing host survivability.

Along with haematological changes evaluation of histological images are the key ingredients in medical diagnosis and prognosis. Evaluation of histopathological changes is commonly used for detecting organ-specific effects related to chemical exposure<sup>52</sup>. The liver and kidney are important organs of metabolism, detoxification, storage and excretion of xenobiotics and their metabolites, and are especially vulnerable to damages. The evaluation of kidney and liver histopathological changes have played a crucial role in studying the toxicity effects of a drug<sup>53,54</sup>. Cisplatin-induced nephrotoxicity, recognized as the most important dose-limiting factor, has been suggested to be caused probably due to apoptosis, inflammatory mechanism, and generation of ROS<sup>53</sup>. In the present study, cisplatin caused the destruction of the renal tubular cells represented by glomerular atrophy, infiltration of cells and tubular congestions and destruction (Fig. 5). Cisplatin treatment developed severe hepatotoxicity causing diffused sinusoidal distortion, congestion in the central vein and remarkable locational hepatocytes damage (Fig. 6). Different studies have suggested that increased production of ROS and oxidative stress play an important role in cisplatin-induced liver damage resulting in a reduction in the mitochondrial membrane potential and a decrease in antioxidant enzymes<sup>55</sup>. The general architecture of the liver, the

appearance of the hepatocytes, the hepatic sinusoids, portal triads, and central veins showed that MeOH-propolis treatment develops no adverse effects in the hosts (Fig. 6). Other studies also observed that the ethanolic extract of propolis did not produce any toxic effect in BALB/c mice showing the normal histological architecture of the liver, spleen, kidney, and brain after propolis treatment<sup>51</sup>.

Sperm abnormality (SA) analysis is considered feasible and reliable endpoints to identify chemicals that induce spermatogenic dysfunction. The abnormalities in sperm morphology have been suggested to be a consequence of mutagenic effects. Various reports have shown the cisplatin-induced reproductive toxicity in male rats and it is correlated with an increase in the thiobarbituric acid reactive substances (TBARS) and oxidative stress in testicular tissues, and change of the intra-testicular testosterone concentrations<sup>56</sup>. In the present study also sperm abnormality analysis showed that cisplatin-induced various types of sperm abnormalities in DL-bearing mice with amorphous head and hookless sperm occurring more frequently (Table 5; Fig. 7 & 8). However, in MeOH-propolis- treated tumor-bearing mice the frequency of various abnormalities in sperms was very low (Table 5; Fig. 8) which suggests that MeOH-propolis did not develop sperms related mutagenic effects in the hosts. Propolis has been reported to improve sperm characteristics of rabbit reproductive toxicity induced by an endocrine disruptor, trypheniltin<sup>57</sup>. It has also been shown that propolis ameliorates the toxicity on sperm quality and reproductive organs of cyclosporine-A, an immunosuppressive drug, possibly by scavenging the free radicals and increasing the antioxidant activities<sup>58</sup>. In present studies also the increasing antioxidant activities in the form of GSH levels were noted in the liver and kidney after MeOH-propolis treatment (Table 2) which may infer that the same thing should be happening in the testes also to involve similar mechanism to keep away any testicular toxicity in tumor-bearing hosts. It has been demonstrated that ethanol-extracted Brazilian green propolis did not induce significant histopathological changes in any organ, and further exerted anti-inflammatory and antitumorigenic effects increasing the survival of Wistar Hannover rats<sup>59</sup>. MeOH-propolis has also been found to exhibit antioxidant activity because of the presence of polyphenols such as flavonoids and phenolic acids<sup>60</sup>. The

antioxidant activity of propolis has been reported to display these beneficial effects by suppressing the formation of free radicals to show various health-promoting useful effects.

## Conclusion

In conclusion, the present findings demonstrate the combined action of the MeOH-propolis as an anticancer as well as its ability to stabilize various toxicity parameters in cancer-bearing mice. Thus, the possibility of using propolis as a natural alternative to chemotherapy to avoid the side effects and supplementation of the bee products including propolis in food should have important beneficial effects in the body.

## Conflict of interest

The authors declare no potential conflict of interest concerning the authorship and/or publication of this article.

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